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The Three *Bacillus anthracis* Toxin Genes Are Coordinately Regulated by Bicarbonate and Temperature

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The two *Bacillus anthracis* toxins are composed of three proteins, protective antigen, lethal factor, and edema factor. The structural genes for these three components are located on the virulence plasmid pXO1. We constructed transcriptional fusions between the regulatory region of each of these genes and *lacZ*. Each construct was then inserted as a single copy at the corresponding toxin gene locus on pXO1, resulting in three isogenic strains. Two environmental factors, bicarbonate and temperature, were found to induce β-galactosidase synthesis in each recombinant strain. Furthermore, the transcription of the three toxin genes appears to be coordinately regulated.

Bacillus anthracis is the gram-positive, spore-forming bacterium responsible for anthrax. The major virulence factors of *B. anthracis* are a poly-γ-D-glutamic acid capsule and two toxins composed of three proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF). The genes encoding PA, EF, and LF, designated *pag*, *cya*, and *lef* (2, 7, 23), respectively, are located on the 185-kbp virulence plasmid pXO1 (14) and appear to be distinct transcriptional units (20).

In an infected animal, anthrax is characterized by the massive proliferation of capsulated bacilli and the production of the two anthrax toxins (11). In vitro, toxin synthesis and capsule formation are induced by bicarbonate under specific culture conditions. Previous work, using quantitative RNA analysis, demonstrated that the expression of *pag* was modulated by bicarbonate at the transcriptional level and that additional factors encoded by pXO1 were also required (1, 4). A similar role for pXO1 in expression of the *cya* gene has been demonstrated (4). Recently, Uchida and coworkers cloned a gene from pXO1, *atxA*, which is involved in the *trans*-activation of *pag* and enhances the synthesis of EF and LF (22). Furthermore, Koehler et al. showed that *pag* transcription is initiated at two different promoters, P1, which is both bicarbonate and *atxA* dependent, and P2, which is a weak constitutive promoter (9).

Quantitative studies of *pag* gene expression using *B. anthracis* strains harboring transcriptional fusions in *trans* have been conducted previously (1, 3). It has been shown that bicarbonate activates *pag* expression throughout the exponential growth phase and maximally in the late log phase. However, the use of autonomously replicating plasmids in these studies presents some disadvantages, since the plasmid copy number and supercoiling, among other things, may be different from those of pXO1.

In the present work, we constructed *B. anthracis* strains

TABLE 1. Plasmids and strains

Plasmid or strain	Relevant characteristic(s)	Source or reference
Conjugative plasmids		
pBALA200 and 201	Conjugative *lacZ* cloning vectors, replicative in *B. anthracis*; Spc^r	8
pAT112	Conjugative-suicide (in *B. anthracis*) vector; Erm^r Kan^r	21
pBALA140	*pag-lacZ* fusion in pAT112	This work
pBALA143	*lef-lacZ* fusion in pAT112	This work
pBALA144	*cya-lacZ* fusion in pAT112	This work
B. anthracis plasmids		
pXO1	185-kbp *B. anthracis* virulence plasmid encoding toxin components PA, EF, and LF	14
$pXO1_{RBAF140}$	Recombinant pXO1 with pBALA140 integrated at the *pag* locus	This work
$pXO1_{RBAF143}$	Recombinant pXO1 with pBALA143 integrated at the *lef* locus	This work
$pXO1_{RBAF144}$	Recombinant pXO1 with pBALA144 integrated at the *cya* locus	This work
B. anthracis strains		
7702	Sterne strain ($pXO1^+$)	Pasteur Collection
RBAF140	Sterne strain derivative containing $pXO1_{RBAF140}$	This work
RBAF143	Sterne strain derivative containing $pXO1_{RBAF143}$	This work
RBAF144	Sterne strain derivative containing $pXO1_{RBAF144}$	This work

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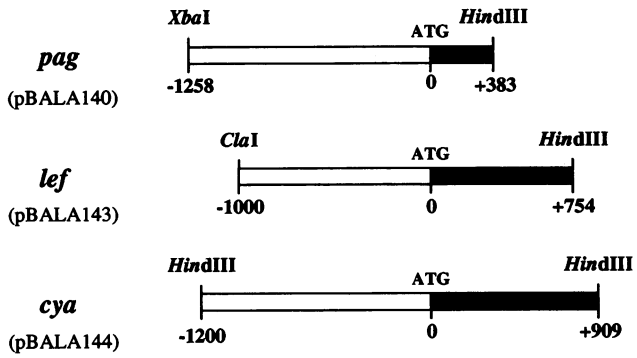

FIG. 1. Regulatory regions of *pag*, *lef*, and *cya* used in the construction of the transcriptional fusions. The DNA fragments encompass coding regions (solid bars) and upstream regulatory regions (open bars). Numbers underneath the bars indicate nucleotide positions relative to the translation initiation codon (ATG). The numbers on the 5′ ends of the *lef* and *cya* fragments are approximate. The name of the suicide plasmid carrying the respective fusions is indicated in parentheses for each gene.

carrying transcriptional fusions integrated as monocopies by a single crossover event in their corresponding loci on pXO1. The effects of bicarbonate, growth phase, and temperature on the expression of these fusions have been studied, and our results suggest that these genes are coordinately regulated.

Construction and characterization of recombinant *B. anthracis* strains. In order to select directly for recombinant *B. anthracis* strains carrying single-copy fusions on pXO1, we used a suicide vector lacking a gram-positive origin of replication (pAT112) (21). Unless otherwise noted, genetic manipulations were conducted in *Escherichia coli* JM105 or *B. anthracis* 7702 Sterne. *E. coli* and *B. anthracis* were grown at 37°C in L medium and brain heart infusion medium, respectively. Antibiotics were used at the following concentrations for *B. anthracis* and *E. coli*, respectively: erythromycin, 5 and 180 μg/ml, and kanamycin, 20 and 40 μg/ml. Spectinomycin was used at 60 μg/ml for both bacteria.

The DNA fragments containing the putative regulatory regions of *pag*, *cya*, and *lef* were fused to the *lacZ* reporter gene in pBALA200 and 201 (8) (Table 1 and Fig. 1). The resulting transcriptional fusions *pag-lacZ*, *lef-lacZ*, and *cya-lacZ* were subcloned in pAT112 (21), giving rise to pBALA140, pBALA143, and pBALA144, respectively (Table 1 and Fig. 1). These plasmids were transferred directly from *E. coli* JM83(pRK24) to *B. anthracis* 7702 by mating, as previously described (17, 21). Recombinant *B. anthracis* organisms were selected for erythromycin resistance and appeared at a low frequency (10^{-9} to 10^{-8} recombinants per *E. coli* donor). Recombinants were expected to result from a single crossover event between the regulatory region present on the pAT112 derivative and the region present on pXO1 at the original locus (Fig. 2A). This type of event would lead to the integration of the entire suicide plasmid, creating a recombinant clone in which the *lacZ* gene on pXO1 is preceded by the upstream regulatory region of the wild-type gene (18). The toxin structural gene is still present in such a clone and is preceded by the cloned regulatory region (Fig. 1).

Recombinant pXO1 plasmids recovered from strains carrying the integrated transcriptional fusions showed the expected patterns in restriction enzyme analysis. Southern blot hybridizations, to verify that the suicide plasmids had integrated

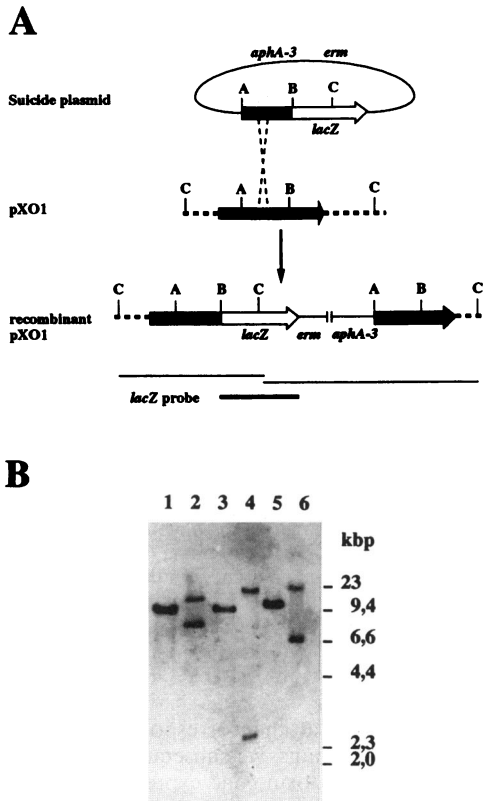

FIG. 2. Schematic diagram for the construction of the isogenic fusion strains RBAF140, RBAF143, and RBAF144 and Southern blot analysis. (A) Integration of the suicide fusion plasmid on pXO1. Recombination occurred between the A-B fragment of the suicide plasmid and the homologous region on pXO1. The designated crossover points are arbitrary. The A sites represent the restriction sites *Xba*I, *Cla*I, and *Hin*dIII in the case of the *pag*, *lef*, and *cya* genes, respectively. B represents a *Hin*dIII site in all three genes. C corresponds to a unique site in the *lacZ* gene (*Sac*I in pBALA140 and pBALA144 and *Cla*I in pBALA143) and sites outside the integration locus on pXO1. The lines at the bottom of the panel represent the fragments expected to hybridize with the *lacZ* probe if the recombinant pXO1 plasmids were cut at C. *erm*, erythromycin resistance gene; *aphA-3*, kanamycin resistance gene. (B) Autoradiogram of a Southern blot with a *lacZ*-specific probe. Lane 1, pBALA140 cut with *Sac*I; lane 2, $pXO1_{RBAF140}$ cut with *Sac*I; lane 3, pBALA143 cut with *Cla*I; lane 4, $pXO1_{RBAF143}$ cut with *Cla*I; lane 5, pBALA144 cut with *Sac*I; lane 6, $pXO1_{RBAF144}$ cut with *Sac*I.

correctly, were performed with restriction enzymes that cut within the fusion only once (Fig. 2A). Two hybridization signals at the expected sizes were observed, and the absence of a signal comigrating with the linearized recombinant plasmid indicated that in each case a single copy had been inserted at the corresponding locus (Fig. 2B). This was subsequently confirmed by subcloning of DNA fragments encompassing the crossover regions. In order to analyze the stability of the constructs, the three fusion strains were grown for 16 h in brain heart infusion medium in the absence of selective pressure. Revertants which had deleted the integrated fusion appeared at a frequency of less than 3%.

Our data show that the recombinant strains RBAF140, RBAF143, and RBAF144 are the results of the integration of single copies of the *pag-lacZ*, *lef-lacZ*, and *cya-lacZ* suicide

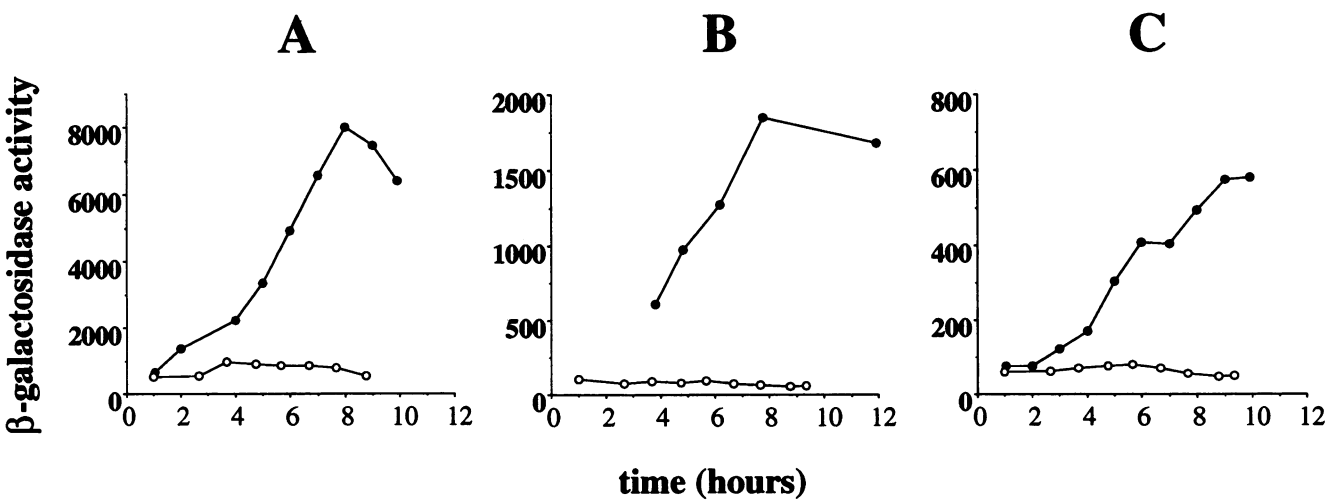

FIG. 3. Effect of bicarbonate on β-galactosidase synthesis by RBAF140 (A), RBAF143 (B), and RBAF144 (C). Strains were grown at 37°C in R medium with (●) or without bicarbonate (○). The growth curves are similar to those shown in Fig. 4. β-Galactosidase activities are expressed in Miller units (15). Note the differences in the ordinate scale among the three strains. Experiments were conducted at least twice, and enzymatic assays were performed in duplicate. The curves represent results from a representative experiment.

plasmids, respectively, at the corresponding loci on pXO1. Using these isogenic strains, we subsequently conducted studies of the regulation of toxin gene expression.

Effect of medium composition. Medium composition, including the presence or absence of bicarbonate, is known to influence the synthesis of the three anthrax toxin components (19), and this regulation occurs at the level of transcription for *pag* (1). Thus, we used our three transcriptional fusion-carrying strains to examine toxin gene expression in various media.

Analysis was initially carried out during growth at 37°C in synthetic R medium (19) in the presence or absence of bicarbonate (Fig. 3). For these experiments, *B. anthracis* strains were incubated for 16 h in a 5% CO_2 atmosphere on nutrient broth plates containing yeast extract (0.3% [wt/vol]), horse serum (10% [vol/vol]), and sodium bicarbonate (0.8% [wt/vol]). Cells were scraped from plates into either R medium containing 0.4% (wt/vol) sodium bicarbonate (R/bicarbonate), in tightly closed screw-cap flasks, or R medium without sodium bicarbonate (R/air), in loosely closed flasks, to obtain an initial optical density at 600 nm of 0.05, and cultures were incubated with slow shaking (80 rpm). The cell growth rates and final pHs under the two sets of culture conditions did not differ significantly. β-Galactosidase activity was assayed as described by Dingman et al. (6). An increase in β-galactosidase synthesis in the presence of bicarbonate was observed for all three strains (Fig. 3). The induction of fusion expression by bicarbonate was observed throughout the exponential growth phase and was maximal during late log phase. The ratios between the levels of β-galactosidase synthesis in R/bicarbonate and those in R/air were found to be similar for the three strains (8, 18, and 10 for *pag-lacZ*, *lef-lacZ*, and *cya-lacZ*, respectively), as deduced from Fig. 3. These results indicate that *lef* and *cya* are regulated at the transcriptional level by bicarbonate and confirm the bicarbonate induction of *pag* expression. In addition, our data suggest that the three toxin genes are coordinately regulated.

Under inducing conditions, the level of β-galactosidase produced by RBAF140 is five times greater than that produced by RBAF143 and ten times that produced by RBAF144 (Fig. 3). A *B. anthracis* Sterne strain grown under equivalent conditions produces approximately 20 μg of PA, 5 μg of LF, and 1 μg of EF per ml of culture (10). Therefore, the relative levels of β-galactosidase production among the three fusion-carrying strains appear to reflect the relative quantities of the corresponding toxin proteins produced by the parental strain. If we make the assumption that the mRNAs of the three fusions are equally stable, this observation supports the notion that the regulation of toxin synthesis by bicarbonate occurs primarily at the level of transcription.

The role of medium composition was also analyzed. RBAF140, RBAF143, and RBAF144 grown in brain heart infusion medium (16 h, 37°C) all produced less β-galactosidase than they did when they were grown in R/air medium. Therefore, medium components other than bicarbonate also affected toxin gene expression.

The effect of bicarbonate in culture medium may be compared with its physiological role. Bicarbonate is found in vivo, where it is in equilibrium with CO_2 and contributes to the buffering of extracellular fluids, at concentrations ranging from 15 to 40 mM (5). For this reason, the concentration of bicarbonate is a good indicator of the host environment. In our cultures, the bicarbonate concentration was 48 mM, close to that found in vivo. Therefore, bicarbonate may play similar roles in vivo and in vitro by providing an optimal environment for expression of the *B. anthracis* toxin genes.

Effect of temperature. Previous reports have described the difference in the susceptibilities of warm- and cold-blooded animals to anthrax. Since temperature is not a critical factor for germination, we hypothesized that it could be important for toxin production. We investigated the β-galactosidase expression of RBAF140, RBAF143, and RBAF144 grown in R/bicarbonate at 28°C (low temperature) or 37°C (high temperature). At 28°C, the levels of β-galactosidase synthesis among all three strains were 4 to 6 times lower than those observed at 37°C (Fig. 4). Therefore, transcription of the three fusions is enhanced at high temperature by the same factor. In order to refine these results, temperature shift experiments were conducted (Fig. 4). When cells were grown at 28°C, β-galactosidase synthesis was not induced. Shifting the cultures to 37°C,

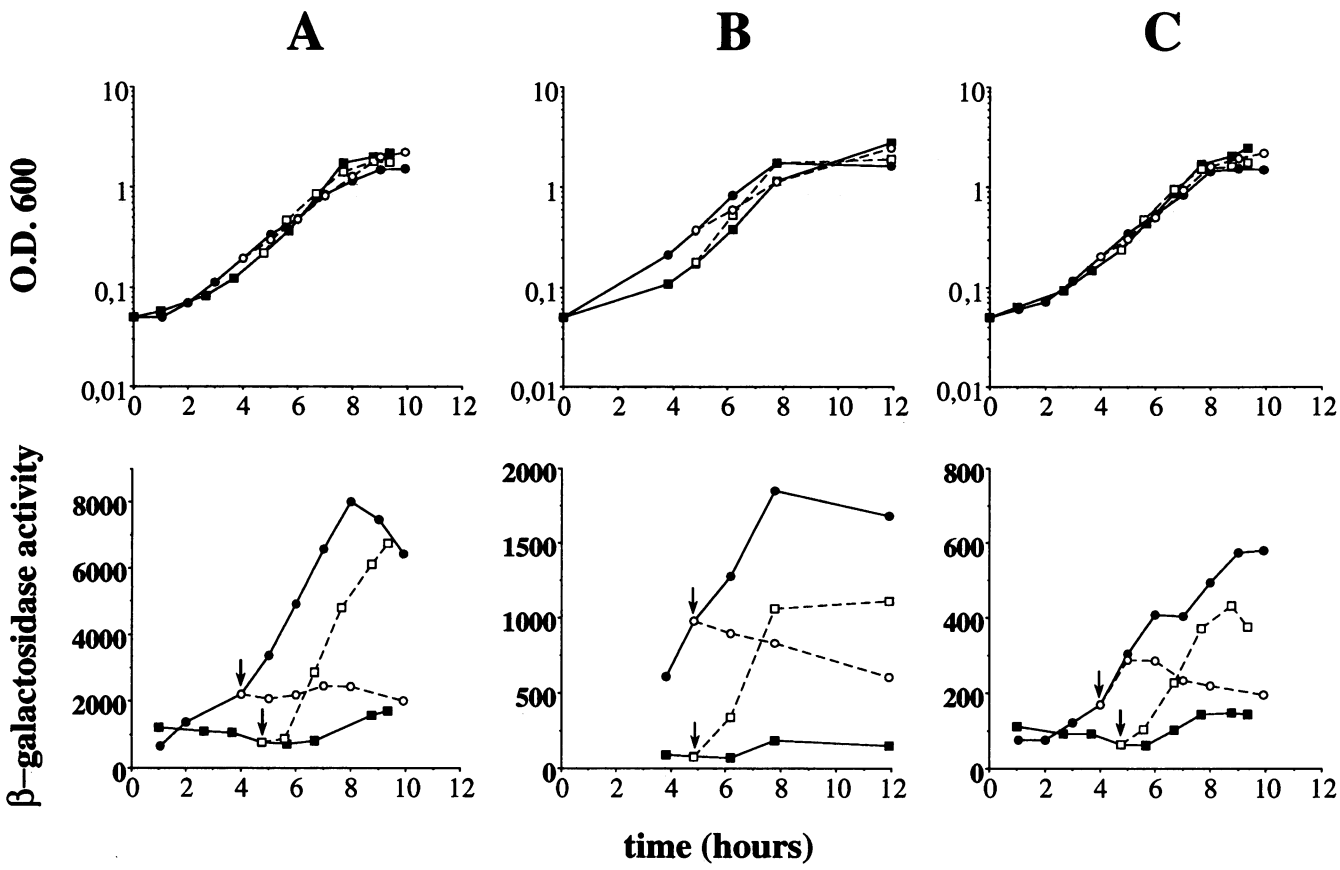

FIG. 4. Effect of temperature on β-galactosidase synthesis by RBAF140, RBAF143, and RBAF144. Strains were grown in R/bicarbonate in tightly closed screw-cap flasks. Growth curves (upper panels) and β-galactosidase specific activity curves (lower panels) for RBAF140 (A), RBAF143 (B), and RBAF144 (C) are shown. Cultures were grown at 37°C (●) or at 28°C (■) and shifted, where indicated (arrows), from 37 to 28°C (○) or from 28 to 37°C (□). β-Galactosidase activities are expressed in Miller units. Note the differences in the ordinate scale among the three strains. Experiments were conducted at least twice, and enzymatic assays were done in duplicate. The curves represent results from a representative experiment. O.D. 600, optical density at 600 nm.

however, led to a rapid increase in β-galactosidase specific activity in each strain. Conversely, in cultures shifted from high to low temperature, enzyme synthesis leveled off, while it continued to increase in unshifted cultures. Changes in the transcription levels (i.e., turning transcription off or on) when the temperature was shifted occurred very rapidly. Thus, temperature seems to be an important signal for the regulation of toxin production.

Anthrax is a disease primarily of mammals. In 1884, Metchnikoff observed that frogs and lizards were not normally susceptible to anthrax (12). In contrast, if these reptiles were kept at 35 to 37°C and then infected with virulent bacilli, they were killed. Considering the fundamental role of toxin in anthrax, the transcriptional regulation of toxin gene expression by temperature may explain these old observations. A shift from ambient temperature to 37°C has been shown to induce the synthesis of virulence factors in other pathogenic bacteria, including *Shigella flexneri* and *Bordetella pertussis* (for a review, see reference 13). Nevertheless, virulence genes are not necessarily optimally expressed at 37°C, as exemplified by the ToxR regulon of *Vibrio cholerae* (16). The identification of temperature as an environmental factor in *B. anthracis* toxin synthesis is therefore an important observation.

It should be emphasized that in each fusion strain described in this work, one of the three toxin structural genes is preceded by the corresponding cloned regulatory region (Fig. 1 and 2A). The production of each toxin component, in response to bicarbonate and temperature, was found to be the same in the recombinant and in the parental Sterne strains (data not shown). This suggests that the regulatory regions used in the construction of these strains are sufficient for the proper expression of the corresponding genes in vitro (Fig. 1).

The coordinate regulation of the three toxin genes may be achieved through a common transcriptional regulator or the coordinated responses of different regulators. It has been shown that both a positive and a negative *trans*-acting regulator were required for transcription of *pag*. Uchida et al. (22) have isolated a gene, *atxA*, whose product is a *trans*-activator of *pag* expression. Their results also suggest that AtxA enhances the synthesis of LF and EF, supporting the common *trans*-activator hypothesis. Nevertheless, some important questions remain. For example, the DNA sequences of regulatory targets are often conserved. However, sequence or secondary structure similarities have not been found in the presumed regulatory regions of the three toxin genes. Is the putative *pag* negative regulator also common to the three genes? These questions await further molecular analysis.

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